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(54) Title: **SUBSTANCES OF POLYPEPTIDE NATURE USEFUL IN HUMAN THERAPY**

(57) Abstract

Substances of polypeptide nature obtainable by extraction from animal tissue homogenates, which substances can induce, when administered to various animal species, the formation of antibodies able to recognize *in vivo* or *in vitro* antigens which are present in human tumoral cells; and can decrease or inhibit pain, induce an effect of cell lysis, and inhibit or slow down the tumor growth, when administered to humans affected by malignant tumors of different kinds.

\* See back of page

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SUBSTANCES OF POLYPEPTIDE NATURE USEFUL IN HUMAN THERAPY

The present invention concerns substances obtainable by extraction of animal tissues, particularly from goat or ovine tissues, which substances are useful in therapy and in diagnostics. More particularly, the 5 invention refers to substances having surprising biological properties which make them useful in oncological therapy (or at least in supporting therapy of the neoplastic disease) and in diagnostic applications of the immunohistological and immunosero-10 logical kind.

Besides the ongoing research for "exogenous" anti-tumor drugs of synthetic, semi-synthetic, fermentative or extraction origin, more and more attention has recently been paid to a new approach based on the study 15 of physiological compounds, naturally occurring in the body, which are able to inhibit the growth of tumoral cells through direct cytotoxic effects or by means of complex interactions with the humoral or cellular components of the immune system.

Within this trend, remarkable research efforts are paid for instance to interferons, tumor necrosis factor (TNF), cytokines and lymphokines such as interleukins, immunotoxins deriving from conjugates of monoclonal antibodies with cytotoxic substances of different kind, 20 etc.

Poor attention has up to now been paid to the search for compounds which can be extracted from xenogenic tissues. A factor, named AFL, obtainable by

extraction of sheep and lambs embryos, has been studied since 1940 but, apparently, without applicative results worth of further investigation (Schweiz-Rundsch-Med. Prax. 1990 79 (16): 498-502).

5 Starting from previous studies of the inventor on the presence of substances having unusual immunological properties (Biomed. Pharmacother. 1987, 41, 2-5) in the serum of patients affected by tumors, it has now been found that substances of polypeptide nature can be  
10 extracted from animal tissues, particularly from those of goats and sheeps, which substances have the following surprising properties:

- they can induce the formation of antibodies able to recognize human tumor antigens;  
15 - they can decrease or inhibit neoplastic pain and they can induce an effect of cell lysis and inhibit or slow down the tumor growth, when administered to humans affected by malignant tumors of different kinds.

The meaning of the term "substances of polypeptide nature" as used in the present disclosure, should be intended to comprise any kind of polypeptide compound, such as proteins, glycoproteins, mucoproteins, and the like.

25 The substances of the present invention may be obtained by extracting, preferably in acidic conditions, homogenates of animal tissues, particularly of goats and sheeps. The use of goat organs is particularly preferred; preliminary tests do not exclude the use of other animal species, for instance sharks.

30 Extraction tests of liver and intestine confirmed the presence, in these organs, of the substances having

the above reported characteristics; therefore it can be assumed that their distribution is ubiquitous and not limited only to one organ or organ groups. Due to practical reasons, reference is hereinafter specifically made to goat liver, which is more convenient to treat and more easily available than other organs.

The extraction of the substances of the invention comprises the following steps:

10 a) homogenization of tissues and organs with usual techniques;

b) treatment of the homogenates with strong inorganic acids at temperatures lower than 10°C, or equivalent extractive methods (detergents, urea, etc.);

15 c) centrifugation and dialysis against water;

d) treatment with hypertonic solution, centrifugation or membrane filtration and dialysis against water and then against saline buffer (PBS);

20 e) ultrafiltration on membranes having cut-off 10,000 D, up to a concentration of about 1 mg/ml of proteins;

f) optional further purification by gel-chromatography.

In step b) 2N perchloric acid at a temperature of 25 about 4°C is preferably used.

In step d) a 3M KCl solution is preferably used and treatment is carried out for about 24 hours at 4°C. The dialysis, filtration or centrifugation operations are carried out according to usual methods.

30 The obtained substance may directly be used according to the invention.

It will accordingly be treated with usual methods to make it sterile and apyrogenic for the administration to patients or animals in form of suitable pharmaceutical compositions, such as those disclosed in  
5 Remington's Pharmaceutical Sciences Handbook, Mack Pub. Co., N.Y., USA.

Examples of said compositions comprise vials of lyophylized active principles which can be dissolved prior to the use with sterile saline solutions, solutions or suspensions in aqueous or oily sterile solvents and similar compositions suitable for the in vivo administration.  
10

Preliminary clinical tests, hereinafter summarized, allow to assume that the substances of the invention may be administered in a wide range of doses, for instance from 0,1 to 100 mg/day for more consecutive days or at different intervals (e.g. once a week, once a month or even longer periods). Particularly, it has been noticed that using substances extracted from goat  
15 liver, hereinafter referred to as LGE, a single administration of an amount of 1-3 mg of dry substance (1-3 ml of solution) can be sufficient to obtain surprising and very fast results in patients affected by tumor pathologies.  
20

A second administration after about 30 days may sometimes produce advantageous effects. It cannot however be ruled out that the ongoing tests could ask for changes in posology and treatment schedule, determined according to the pathology and the patient's  
25 conditions.  
30

The following example further illustrates the in-

vention.

EXAMPLE

100 g of male goat liver were homogenized in a blade homogenizer, the homogenate was re-suspended in  
5 distilled water to a final volume of 400 ml.

400 ml of 2N  $\text{HClO}_4$  were dropped into said suspension in about 20 minutes at 4°C and stirring for further 30 minutes. After centrifugation at 10,000 g for 20 minutes, the precipitate was discarded and the  
10 supernatant was dialyzed against tap water and then against distilled water.

After dialysis, powdered KCl was added to the obtained liquid till obtaining a 3 molar solution and the mixture was stirred for 24 hours at 4°C. After  
15 centrifugation at 100,000 g for 1 h., the supernatant was dialyzed against distilled water and phosphate buffer (PBS).

The obtained solution (800 ml) has a mean protein concentration of 200  $\mu\text{g}/\text{ml}$  according to the Lowry  
20 method.

The solution was concentrated by ultrafiltration to a concentration of 1 mg/ml, it was named LGE and directly used for the clinical tests, hereinbelow summarized, making always reference to the abbreviation  
25 LGE.

This product was assayed in PAGE 4/30 where 4 main bands were evident (Fig. 1): from the calibrating curve obtained from the parameters of the standards, the molecular weights of the 4 main bands were calculated:

30        1<sup>st</sup> band: = about 50,000 d  
            2<sup>nd</sup> band: = about 20,000 d

3<sup>rd</sup> band: = about 14,800 d

4<sup>th</sup> band: = about 12,000 d

As a purification step subsequent to the LGE extraction, a ion-exchange chromatography was used, according to the following method. About 20 ml of LGE were dialyzed against a phosphate buffer ( $\text{Na}_2\text{HPO}_4$ - $\text{NaH}_2\text{PO}_4$  0.01 M, pH 6.5). After dialysis the sample was filtered through a 0.45  $\mu\text{m}$  filter and then changed on a TSK DEAE SPW 7.5x75 mm column, equilibrated in the same dialysis buffer, with a flow of 30 ml/hour.

The column elution was continued for 100 minutes with the same flow and buffer.

A linear gradient of phosphate buffer at pH 6.5 is started from an initial molarity of 0.01M to a final molarity of 0.1M, said gradient having a duration of 100 minutes and the operative flow being kept at 30 ml/hour.

From the eluate, 0,5 ml fractions were collected and the different zones corresponding to the various peaks were pooled.

As it can be derived from the chromatogram (Fig. 2) various protein fractions were obtained which were singularly assayed in PAGE PAA4/30 to evaluate the components thereof. The starting buffer, defined as zone A, and the gradient fraction having the highest protein concentration, defined as zone B, were preliminary considered as more interesting since low molecular weight proteins were mostly present in the zone A whereas the proteins having molecular weight of 50,000 daltons were present in higher concentrations in the zone B.

Those two samples, zones A and B, were subsequently labelled with I<sup>125</sup> (Fig. 3 and 4) and used in RIA. In order to make available to the clinical experimentation, in practically useful amounts, zones A and B to be separately administered, the purification of larger amounts of LGE was required.

A zone preparative gel DEAE-Sephadex column, equilibrated and eluted under the same conditions as the DEAE SPW column, was used in FPLC.

800 mg of LGE were purified by this column and 42 mg of zone A and 15 mg of zone B were obtained.

Subsequently a portion of LGE batch 2/90 was analysed in reverse phase with an Aquapore Butyl (30 x 4.6 mm 7U) of Applied Biosystem, using as eluents water and acetonitrile containing 0.05% TFA in linear gradient of acetonitrile from 25 to 55% in 15 minutes. The chromatogram recorded at 220 nm is shown in Fig. 5. A portion of zone A and zone B samples deriving from DEAE Sephadex was chromatographed in reverse phase under the same conditions reported for crude LGE (Figs. 6 and 7).

Examining the chromatograms of crude LGE and those of zones A and B it could be observed that the more significant protein components found in the raw product are the same in the two samples deriving from zones A and B, even though in different concentrations.

#### Isoelectric point

PAGE Plates LKB 3.5-9.5 were used according to the supplier's instructions.

Unfractionated LGE gave one slight band at pH 8.3, one marked band at pH 6.7-7 and several close bands

between pH 6 and 4.5 having the typical aspect of isoantigens.

LGE zone A gave one marked band at pH 8.2, one marked band at pH 6.7 and some bands in acid zone with 5 a certain prevalence of 3 particular bands at pH 5.6; 5 and 4.9.

LGE zone B showed a remarkable intensity of staining of the zone in the acid zone at pH < 6 with a typical aspect of isoantigens.

10 Biochemical, immunochemical and radioimmunological results

Doses of 5 ml (corresponding to 1 mg of dry extract) were prepared from the solution obtained before the concentration step and were emulsified with 15 Freund's complete adjuvant. 2 Rabbits were immunized with a dose every 15 days.

Of the two original rabbits, one was lost after the first immunization. From the second animal an aliquot of serum (called RF4) was obtained, that was 20 then used for biochemical, immunochemical, radioimmunological and immunocytochemical studies. The RF4 antibody was tested with the Western Blot method against LGE. The antigen was submitted to PAGE SDS PAA 4/30 plates (Pharmacia) and subsequently transblotted 25 into Nitrocellulose. This last was then incubated with RF4 serum and the reaction was revealed with antirabbit peroxydase (PIERCE). As shown Fig. 8, the antibody recognizes proteins of about 50.000 d molecular weight.

A second group of rabbits was immunized with LGE 30 as described above (5 ml, corresponding to 5 mg of dry extract, emulsified with 2.5 ml of Freund's complete

adjuvant).

These rabbits yielded fourteen antisera called anti-LGE 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 which were tested in the same way as RF4.

5 The data obtained from the biochemical methods (in particular, the Western Blot one) suggest that these antisera, unlike the RF4 antiserum, recognized all the proteins components of LGE (Fig. 9).

Moreover, female BALB/c mice 10 week old were  
10 immunized for the production of monoclonal antibodies. Particularly, two mice were immunized with LGE (1 mg/ml) and two were immunized with LGE zone A from DEAE 5PW (1 mg/ml), by subcutaneous administration of 100 µl of antigen + 100 µl of Freund's Adjuvant.

15 The immunization scheme is reported hereinbelow:

1<sup>st</sup> administration: 100 µl of Ag + 100 µl of complete  
Freund's Adjuvant.

2<sup>nd</sup> administration: 100 µl of Ag + 100 µl of complete  
Freund's Adjuvant.

20 7 days after the 1<sup>st</sup> administration  
3<sup>rd</sup> administration: like 2<sup>nd</sup>.

7 days after the 2<sup>nd</sup> administration  
4<sup>th</sup> administration: like 3<sup>rd</sup>.

25 7 days after the 3<sup>rd</sup> administra-  
tion.

At the end of immunizations an aliquot of blood was taken from each mouse and the sera obtained were subjected to the binding test against LGE (Fig. 10) and to immunocytochemical tests.

30 Based on the obtained results, it was decided to sacrifice mouse n. 2, immunized with LGE zone A, in

order to obtain monoclonal antibodies.

Fusion performed according to Milstein gave 250 clones.

A first screening showed that many clones were directed towards the determinants expressed on the LGE low molecular weight components, whereas a lower number of clones recognized the 50.000 d protein. The 2<sup>nd</sup> screening, carried out after clone expansion, gave negative results for all the clones, therefore, it was decided to fuse the spleen from another immunized mouse. The fusion gave 400 clones (100%). In the first test, after approximately 10 days, 64 clones proved to be positive on LGE.

The supernatant of these 64 clones was subsequently tested on LGE, LGE zone A and LGE zone B. The screening showed 4 clones to exhibit a strong binding to 3 antigens, more evident on LGE zone A. These 4 clones were cloned and then retested in the same way on the three antigens. In parallel, the clones were also tested on LGE in Western Blot.

The results from these two tests led us to expand and produce the ascites of 9 clones. The obtained ascites were titrated with the binding test against LGE, LGE zone A and LGE zone B. All the ascites exhibited a good degree of binding to the three antigens, with a certain prevalence for LGE zone A.

On the contrary, the Western Blot test showed that only one monoclonal antibody was able to bind a 50.000 d band, while all the other ones recognized a band with a molecular weight slightly exceeding 50.000 d. At variance with the binding test, none of these

monoclonal antibodies showed any binding to the low molecular weight components. A study with "nude" mice subjected to tumor implantation (human colon adenocarcinoma cell line named HT29) was carried out.

5        10.000.000 Cells in 0.5 ml of medium were inoculated into each animal. Seventeen days after inoculation the animals were treated with the RF4 antiserum previously purified by ion exchange, in order to isolate the immunoglobulin fraction, and then  
10      radiolabelled with <sup>125</sup>I. Each animal received 10.000.000 cpm in 0.5 ml PBS + 1% BSA intraperitoneally.

15      The animals were sacrificed after 24, 48 and 72 hours and necropsied to calculate the antibody localization index.

The capability of antigenic recognition by the sera of LGE immunized rabbits was tested on human tissues.

20      The antisera were tested on human tissue sections with the ABC immunoperoxidase and the immunogalactosidase procedures, and with RIA on human tissue extracts.

25      Sections of formalin fixed - paraffin embedded carcinomas of stomach, large intestine, breast, lung, prostate and ovary, as well as tumoral explants (obtained in nude mice) of HT29 cells (from an intestinal carcinoma), breast cancer MCF7 and GTL16 cells were employed. For control different human normal tissues were used.

30      From the obtained results, it could be evidenced the followings:

- a) Rabbit anti-LGE sera, stained in brown,

recognized superficial and cytoplasmic antigenic structures in neoplastic cells, with variable degrees of titre and specificity, in cases of gastric, large bowel and mammary carcinomas and HT29, GTL16 cells.

5 b) No reaction occurred between the rabbit anti-LGE sera and human normal liver cells in immunocytochemistry and with CEA in immunocytochemistry and RIA.

c) No all tested tumors were positive.  
10 The LGE, the <sup>125</sup>I LGE, the rabbit anti-LGE and the rabbit <sup>125</sup>I anti-LGE systems were tested in vivo, in nude mice and in nude mice xenografted with HT29 cells.

The results obtained were the following:  
15 1) In both groups, <sup>125</sup>I LGE showed in vivo a very rapid turnover; 24-48 hours after subcutaneous injection all radioactivity was localized in urinary system.

20 2) The LGE and the anti-LGE immunoglobulins induced no reduction of the tumor burden in nude mice, even if in same cases a marked hyperaemia of the tumor was observed.

25 3) <sup>125</sup>I-labelled anti-LGE immunoglobulins showed a significant LGE localization in the neoplastic mass 3-5 days after injection in nude mice xenografted with HT29 cells. The above results reproduced in vivo the immunocytochemical data.

The extract (LGE) and the anti-LGE sera were tested in vitro on:

30 1) Primary cultures of human neoplastic pleural effusions.  
2) Neoplastic cell lines: human (lines HMF7 and HT29) and rat (R3230Ac).

## 4) Basophil Degranulation Test.

The results were the following:

1) LGE and anti-LGE sera showed no direct toxic effect on neoplastic cells.

5 2) LGE and the anti-LGE sera showed in vitro no TNF (tumor necrosis factor) activity on target cells.

3) A cytotoxic activity, probably mediated by lymphocytes and/or macrophages, was evidenced on breast cancer cells in primary cultures of neoplastic  
10 effusion.

4) When K562 and HL60 were used as target cells, LGE acted as a strong LAK (Lymphokine-Activated Killer Cells) inducer on human lymphocytes.

This result was variable in comparison with the  
15 IL2 response. In fact lymphocytes from different donors showed different cytotoxic effects when treated with LGE.

On the contrary, LGE and IL-2 together greatly increment this activity (+27.2%).

20 Experiments run with CTLL cells (IL2 sensitive) exclude any type of similarity between LGE and IL-2.

No comparison could be done between LGE and any BRM (biological response modifiers), particularly those of the group 2. In fact LGE was inactive in vitro on tumor cells and in vivo on tumor cells in "nude" mice model. The in vivo response obtained with LGE on the metastatic human pleural effusion indicated that the presence of the immunity mediator cells was necessary.  
25

30 On the other hand, the extraction of LGE in a high acidic medium excluded any similarity with the BRM and with interferons.

5) LGE inhibited "in vitro" the proliferation of PBL. This could be related to a direct inhibitory effect on lymphocytes or it could be mediated through the induction of different inhibitory factors. The 5 inhibitory effects appeared to be dose and time dependent.

LGE was assayed in the basophil degranulation test. This test is used in order to recognize a specific antigen against basophils and/or attached 10 antibodies which have acquired an immune memory. The test is considered positive for values over 30%. On blood samples of a pulmonary carcinoma patient, LGE produced in a very low concentration (1 µg - 0.1 µg/ml) the significant dose dependent basophil degranulation 15 (>80%). Tests performed on further 5 cases showed that 50% patients suffering from neoplasia were positive to this test. No degranulation occurred on controls performed with non washed (i.e. in the presence of circulating antibodies and antigens and not only with 20 factors attached to basophils) and without calcium samples (Tab. 1). Degranulation of basophils is not present using blood of healthy donors.

TABLE 1

5

L.G.E.

		basophils/granulocytes %			% of degranulated B.
10	-whole blood				
	control	25	6050	0.41	
	100µg/ml	21	6000	0.35	15
	10µg/ml	28	6900	0.4	2
	1µg/ml	34	7830	0.43	0
	0.1µg/ml	25	7500	0.34	15
	100µg/ml No Ca++	31	8300	0.37	7
15	-washed blood				
	control	46	8550	0.54	
	100µg/ml	21	10000	0.21	61
	10µg/ml	12	10000	0.12	78
	1µg/ml	9	10000	0.09	83
	0.1µg/ml	25	11000	0.22	59
	100µg/ml No Ca++	43	6300	0.69	0
20	10µg/ml No Ca++	44	6500	0.67	0
	1µg/ml No Ca++	43	6300	0.69	0

25

30

**Toxicology**

Acute and chronic toxicities in animals were tested in mice and rabbits.

For acute toxicity, mice and rabbits were daily 5 subcutaneously injected (for 10 days) with an LGE solution, equivalent to 7.1 mg/kg dose.

Chronic toxicity tests are ongoing in rabbits for six months with weekly doses of 1.2 mg/kg.

No toxic effects neither detectable alterations 10 could be evidenced.

Preliminary studies with  $^{125}\text{I}$  labelled LGE demonstrated a complete clearance in 24/48 hours, through urine.

No toxic effects could be detected by injecting 15 LGE at various concentrations to animals. A letal dose could not be determined.

**Clinical tests****First Phase**

29 Terminal patients were treated on a 20 compassionate basis and with the assent of patients. All of them had previously received multiple anticancer treatments (surgery, radiotherapy, chemotherapy) as well as after supportive treatment, such as courses of anti-inflammatory drugs (FANS and steroids) and opioids 25 or other major analgesics.

Most patients were cachectic and ever precomatous with mean life expectance of < 7 days.

Nevertheless, a positive response was observed in 70% of the patients, the most common effect being the 30 disappearance of pain and the induction in patients of a subjective feeling of well-being; for 48% of the

patients a remarkable increase of life expectance (> 2 months) was observed and no pain recurred.

The reduction of the tumoral mass was observed in 5 on 14 patients survived more than two months (Tab. 5 2).

TABLE 2  
FIRST PHASEMETASTASES OF AND  
NUMBER OF CASES  
RESPONSE TO L.G.E. SUBCUTANEOUS INJECTION (1,5mg)

		POSITIVE 30 days	POSITIVE 15 days	NEGATIVE
GASTROENTERIC	8	3 (37,5%)	1 (12,5%)	3 (37,5%)
LUNG CANCER	6	4 (66,6%)	1 (16,6%)	1 (16,6%)
BREAST CANCER	4	2 (50%)	1 (25%)	1 (25%)
PANCREAS CANCER	4	1 (25%)	2 (50%)	1 (25%)
GENITO-URINARY CANCER	3	3 (100%)	--	--
SARCOMAS	2	--	1 (50%)	1 (50%)
VARIOUS CANCER	2	1 (50%)	--	1 (50%)
TOTAL	29	14* (48,2%)	6 (20,6%)	9 (31,4%)

\* In five cases disappearance of neoplastic masses (clinico-instrumental demonstration)

In the first group of patients, the treatment exerted highly favourable subjective effect, but no further analysis could be done because of the heterogeneity of the pathologic, and clinical 5 conditions, accordingly the patients were not submitted to instrumental or laboratory evaluation.

Moreover the LGE treatments ranged from the daily to the weekly administration (0.025 mg/kg). In this experience no anaphylactic reaction appeared.

10 From this first part of investigations, the following observations were drawn:

- 1) LGE showed no toxicity in man also after repeated daily administration.
- 2) Different batches induced the same clinical 15 effects.
- 3) LGE induced an intense pain reduction with a better anesthesia, appetite and bowel function improvement.

20 In the clinician opinion, it induced an unexplained "euphoria" in very ill patients.

#### Second Phase

During this period 141 new terminal cancer patients were treated. But the attitude of the clinicians was however different basing on the 25 assurance about the harmlessness of the LGE and the need to document the positive effects which were observed. The drug was however still administered only to terminal patients, for whom any other treatment had failed, and on a merely compassionate basis.

30 Almost all patients received a single subcutaneous injection of 1.5 mg LGE (about 0.025 mg/kg). In some

cases (in the last three months) a second dose was administered one month later.

In the following figures, patients were divided in 2 groups defined as "N" and "U".

5 The "N" patient collection represented 108 cases.

The "U" experimentation was performed on 33 patients. The definition of responders was based only on subjective and objective general condition i.e. performance status, as the patients were all with a 10 very low life expectancy.

"U" group (Tab. 3)

After 4 months, 33 terminal ca patients treated with LGE were all alive, among them 11 were considered not yet evaluable and of the remaining 22 patients, 13 15 were responders (3 for a period lower than one month).

Among the remaining 10 responders (> 30 days), 5 were still alive with a life expectancy of 6 months.

The reduction (> 25%) and/or disappearance of tumor mass was shown in this group in 2 patients on 5. 20 The 9 non-responders were all dead.

In the patients of the group "U" controls were performed on 21 patients in different times from LGE administration.

25 In 13 patients the granulocyte increase was statistically significant without any other lymphocytic population increase.

Second phase - "U" Group				
Clinical situation	n° cases	Alive	Dead	regression or disappearance of masses (>25%)
NON RESPONDERS	9	-	9	-
RESPONDERS 30 DAYS	10	5	5	2
RESPONDERS 30 DAYS	3	-	3	-
UNDER EVALUATION	11	11	-	-
TOTAL	33	16	17	2

It is necessary to remind in the evaluation of these data that these were terminal ca patients, highly immunosuppressed, therefore the blood chemistry modification induced by LGE could be hidden or altered 5 by previously performed immunosuppressive therapies (chemio, radiotherapy, ecc.).

"N" Group (Tab. 4 - 5 - 6)

108 Terminal ca patients were treated. Every anti-tumoral specific therapy (chemio, radio, 10 hormonotherapy) was suspended for everyone of these patients as it proved to be ineffective. Patients received only pain therapy, often opioids and FANS, which were interrupted at the beginning of this trial.

All patients received one subcutaneous single dose 15 of LGE (1.5 mg). In some case the dose was repeated 30 days after the first administration.

Only 82 patients out of 108 treated were evaluated (Tab. 4) and among them, after 5 days of LGE treatment, 20 27 (32.9%) were non-responders and 52 (63.4%) were responders.

11 non-responder (13.4%) and 37 (45.1%) responder patients survived within the first 30 days from a single LGE injection (Tab. 4).

TABLE 4  
Second phase - "N" Group

METASTASES OF	nº CASES	nº EVALUABLE CASES	5 DAYS	30 DAYS	- MASSES
GASTROENTERIC CANCER	27	17	7-	1-	4
LUNG CANCER	22	17	5-	5+	
BREAST CANCER	20	16	4-	2-	4
GENITO-URINARY CANCER	12	10	3-	2-	
LARYNX CANCER	3	2	2-	5+	4+
THYROID CANCER	6	4	2-	1-	4
SOFT TISSUE SARCOMAS	4	3	2-	1-	0
BONE SARCOMAS	14	13	2-	1-	1
TOTAL	108	82	27-	11-	4-
			52+	37+	30+

- NON RESPONDERS PATIENTS

+ RESPONDERS PATIENTS

- MASSES: reduction (&gt;25%) or disappearance

After the first month and in the following 4 months, 4 (4.8%) non-responders and 30 responders (36.5%) survived. In 24 (80%) of the 30 responder patients with a survival more than one month also the reduction (> 25%) or the disappearance of the neoplastic mass was shown (Fig. 11 and Tab. 5).

METASTASES OF	nº CASES	NON RESPONDERS		RESPONDERS (>1 month)		- MASSSES*
			-		-	
GASTROENTERIC CANCER	17	1		5		4
LUNG CANCER	17	0		5		4
BREAST CANCER	16	1		8		4
GENTO-URINARY CANCER	10	1		4		4
LARYNX CANCER	2	0		0		0
THYROID CANCER	4	0		1		1
SOFT TISSUE SARCOMAS	3	0		1		1
BONE SARCOMAS	13	1		7		6
TOTAL	82			4		30

\* - MASSSES: reduction (>25%) or disappearance

In these patients, the symptom "neoplastic pain" was studied independently of all other symptoms scored in order to define the patient as responders.

5 Tab. 6 shows the results of this study on 82 patients which confirms the previous observation: in the remarkable percentage of cases, 37/82 (45.1%), the pain often resistant to the most important opioid and to the recent pain therapies, disappeared within 12/24 hours and in any case always within the first days.

10 In an other smaller patient group (24/82), the neoplastic pain disappearance was always complete but it disappeared later (within 30 days).

TABLE 6  
 L.G.E. THERAPY - Disappearance of neoplastic pain  
 METASTASES      n° EVALUABLE      UP TO 5 DAYS      UP TO 30 DAYS  
 OF                  CASES

GASTROENTERIC CANCER	17	9	6
LUNG CANCER	17	9	5
BREAST CANCER	16	9	2
GENITO-URINARY CANCER	10	5	3
LARYNX CANCER	2	0	0
THYROID CANCER	4	2	1
SOFT TISSUE SARCOMAS	3	1	1
BONE SARCOMAS	13	2	6
TOTAL	82	37	24

### Third Phase

Although the patients were controlled following the same criteria listed above, the anatomo-pathologic evaluation of the treatment was introduced. Six 5 patients suffering with different cancers who received a single injection of LGE were submitted to surgery for the exeresis of the tumoral mass after an interval ranging from 4 to 51 days.

A total of 8 specimens was examined, from 6 10 patients.

2 Specimen tissues (both from patients affected by carcinoma of the large intestine) were obtained before treatment.

From the same patients, as well as from other 4 15 patients affected by gastric carcinoma (2 cases), carcinoma of anal tract and of carcinoma of the pancreas, tissues were examined after LGE treatment (injection made from 4 to 51 days before).

Tissues were embedded in paraffin and histologically 20 examined. In addition, immunostaining with monoclonal antibodies against lymphocytes (common leucocyte antigen CLA), PAN P marker (L26 monoclonal), PAN T marker (UCHL1 monoclonal) were performed.

The results indicate that:

25 neoplastic tissues obtained before LGE treatment, showed only a modest inflammatory infiltrate.

The same patients were treated with LGE.

The tissues examined 15 days after LGE treatment showed extensive areas of necrosis and prominent 30 infiltrate of granulocytes.

The same results were obtained in tissues from

different patients 51 days after LGE treatment.

In all cases, independently by the time of examination after LGE treatment, granulocyte infiltration by eosinophilic granulocytes was quite 5 obvious. Staining of different types of lymphocytes showed presence of CLA positive cells, mainly of B type lymphocytes, while UCHL1 cells (T lymphocytes) were rare.

This low number of T lymphocytes combined with the 10 presence of granulocytes and eosinophiles seems to be extremely interesting when compared with haematochemical values of patients in therapy (granulocytes increase during first days after LGE administration) and with the results from PBL cultures 15 in presence of LGE where an inhibition of PBL proliferation has been observed and results of basophil degranulation test.

#### CLINICAL CONCLUSION

The results obtained in 3 multicentric trials were 20 quite homogeneous.

The amount of responders after one month of observation was, respectively, of 48.2%, 45.4% and 45.1% although there was the difference in time, procedure and doctor culture.

25 The overall response after every observation period, including the < 7 days group, was respectively 68.9%, 59% and 63.4%.

An overall response rate (Fig. 12) of 46% (observation period 1 month) and 63.4% of total 30 responders was obtained. It is interesting to remember that in the "N" group (Tab. 4 and 5) 80% of the

responders with more than one month of survival showed a reduction (>25%) or disappearance of tumor mass.

In the same observation period only 4.8% of the non-responders survived.

5 A very unusual and astonishing observation is the dramatic (Tab. 7) disappearance of neoplastic pain also in subjects without response to opioid.

This effect begins before the reduction of tumor mass and it is present also in those patients in which  
10 no objective effect was seen.

TABLE 7  
LGE THERAPY: single subcutaneous injection (1,5mg)  
CLINICAL RESULTS ON 134 PATIENTS

		reduction or disappearance of masses
	< 7 DAYS: 19	-
RESPONDERS	85	30 DAYS: 12
		-
	> 30 DAYS: 54	31
NON RESPONDERS	49	

It is worthwhile to study deeper this clinical observation.

The results of third trial show an intensive granulocytosis and perivascular necrosis in the neoplastic tissue after administration of single dose of LGE and confirm, from an histopathological point of view, the substance activity in inducing the lysis of neoplastic cells in man with a tumor specific mechanism and inducing immunogenic pattern in the host.

CLAIMS

1. Substances of polypeptide nature obtainable by extraction from animal tissue homogenates, which substances can induce, when administered to various animal species, the formation of antibodies able to recognize in vivo or in vitro antigens which are present in human tumoral cells; and can decrease or inhibit pain, induce an effect of cell lysis, and inhibit or slow down the tumor growth, when administered to humans affected by malignant tumors of different kinds.
2. Substances of polypeptide nature obtainable by extraction with  $\text{HClO}_4$  and 3M KCl from animal tissue homogenates, which substances have the following characteristics :
  - molecular weight ranging from 10,000 to 50,000 daltons (by polyacrylamide gel electrophoresis);
  - they can induce the formation of antibodies able to recognize in vivo or in vitro antigens which are present in human tumoral cells, when administered to different animal species;
  - they can decrease or inhibit pain, induce an effect of cell lysis, and inhibit or slow down the tumor growth, when administered to humans affected by malignant tumors of different kinds.
3. Substances as claimed in claim 1 or 2, obtainable from caprine or ovine tissues.
4. Substances as claimed in claim 3, obtainable from goat, sheep or ram tissues.
5. Substances as claimed in claim 1 or 2, obtainable

from shark tissues.

6. Substances as claimed in any one of claims 1 to 5, obtainable from liver or intestine.

7. Substances as claimed in claims 1-2, obtainable from 5 goat liver.

8. Substances of polypeptide nature as claimed in claim 1 or 2, obtainable from goat liver or intestine, which substances have electrophoretic bands on polyacrylamide gel at about 50,000, 20,000, 14,800 and 12,000 daltons.

10. 9. A process for the preparation of the substances as claimed in claims 1 to 7, which process comprises:

a) homogenization of tissues or organs, according to conventional techniques;

15. b) treatment of the homogenates with strong inorganic acids, at temperatures below 10°C, or equivalent extraction procedures;

c) centrifugation and dialysis against water;

20. d) treatment with an hypertonic solution, centrifugation or membrane filtration and dialysis against water and subsequently against saline buffer;

e) ultrafiltration on membranes with cut-off 10,000 to a concentration of about 1 mg/ml of proteins;

f) optionally, further purification by gel-chromatography.

25. 10. Pharmaceutical compositions consisting of, or containing as the active ingredient, the substances of polypeptide nature as claimed in claims 1 to 8.

11. The use of the substances as claimed in claims 1 to 8 as agent useful for the antitumor therapy and for the 30 control of (tumor pathology-related pain).

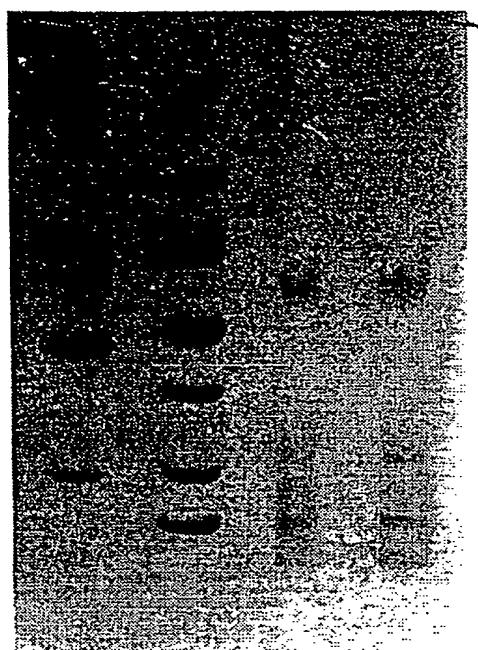
12. The use of the substances as claimed in claims 1 to

8 for the preparation of mono- or poly-clonal antibodies against the antigens present in tumoral cells, for the in vivo therapeutic and diagnostic uses and for the in vitro diagnostic use (RIA, EIA,  
5 immunocytochemistry).

13. The use of the substances as claimed in claims 1 to 8, which substances are suitably conjugated with immuno-modulating substances and/or adjuvants, to produce an active immunity (vaccination) in the healthy  
10 population.

1/12

FIGURE 1

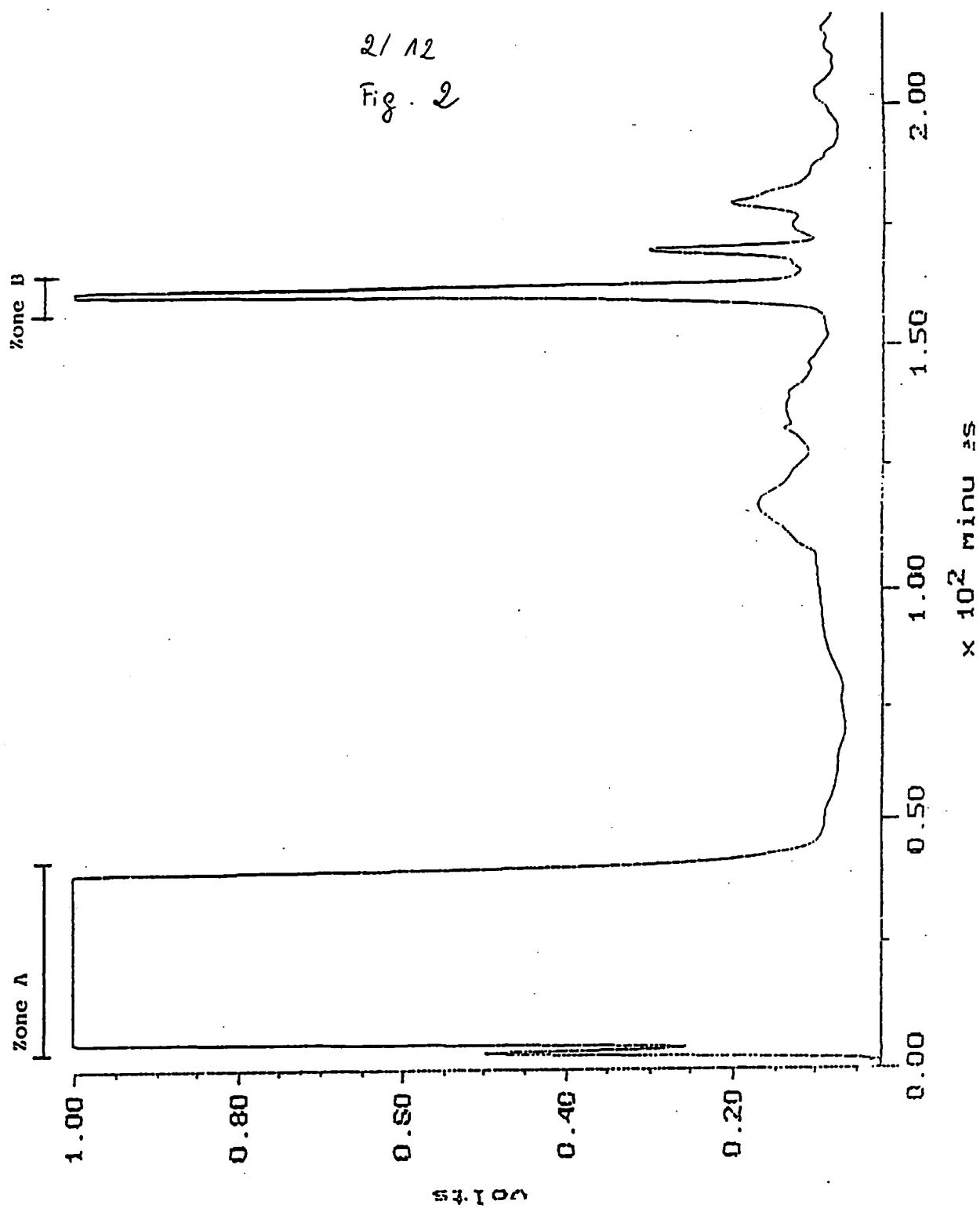


1      2      3      4

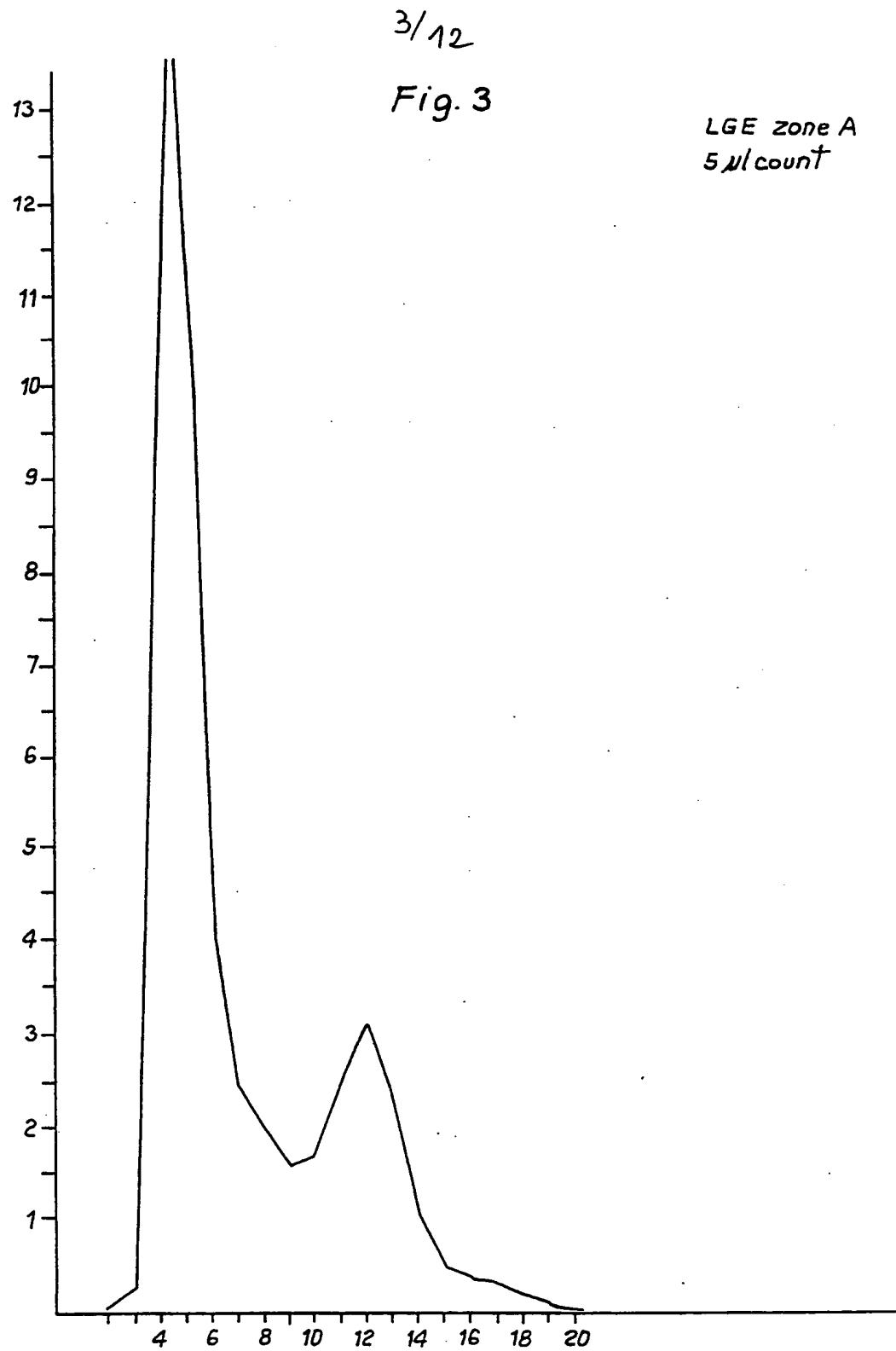
SDS-PAGE in PAA 4/30

- 1: High molecular weight
- 2: Low molecular weight
- 3: LGE Lot. 1
- 4: LGE Lot. 1/B

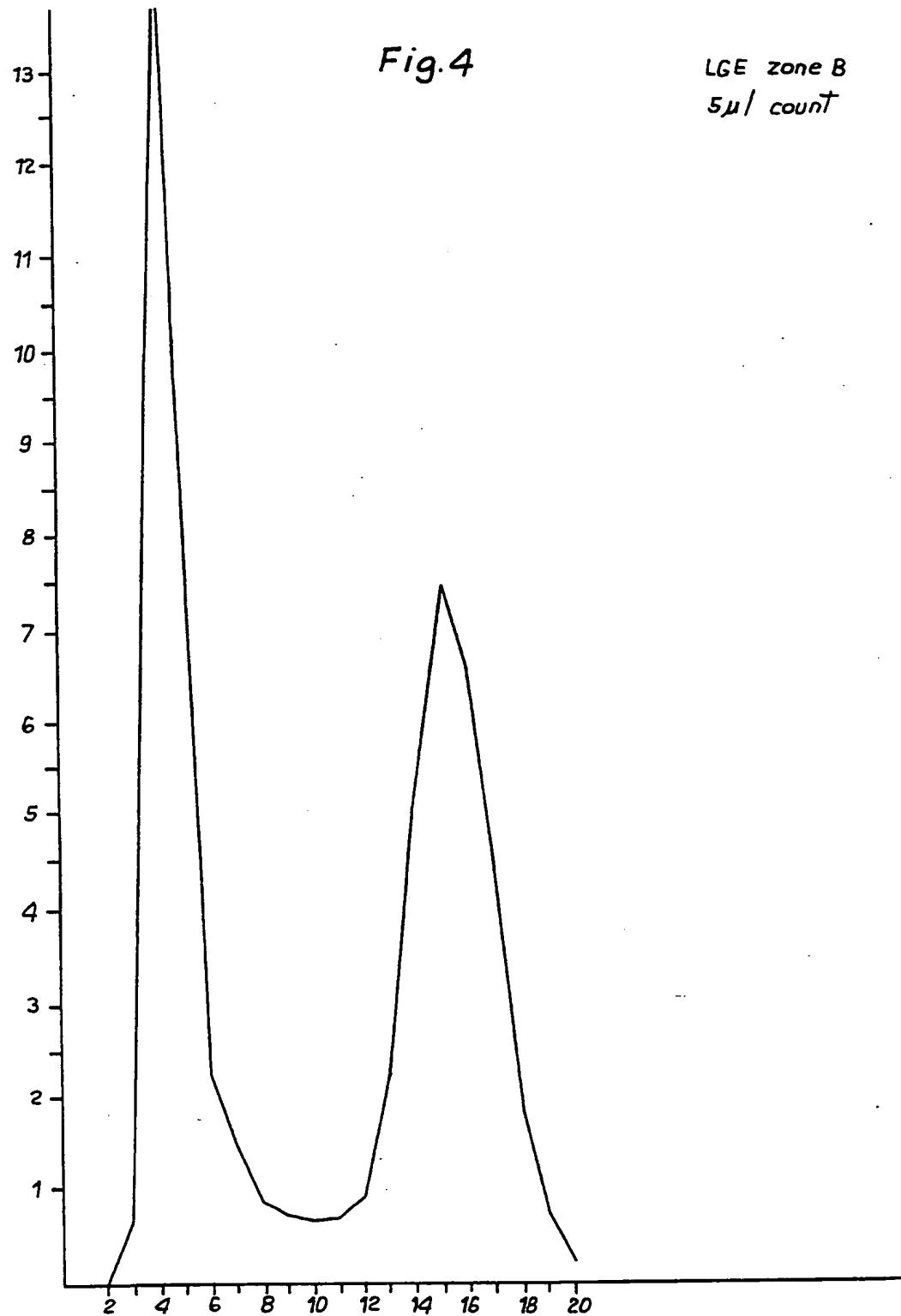
**SUBSTITUTE SHEET**



SUBSTITUTE SHEET

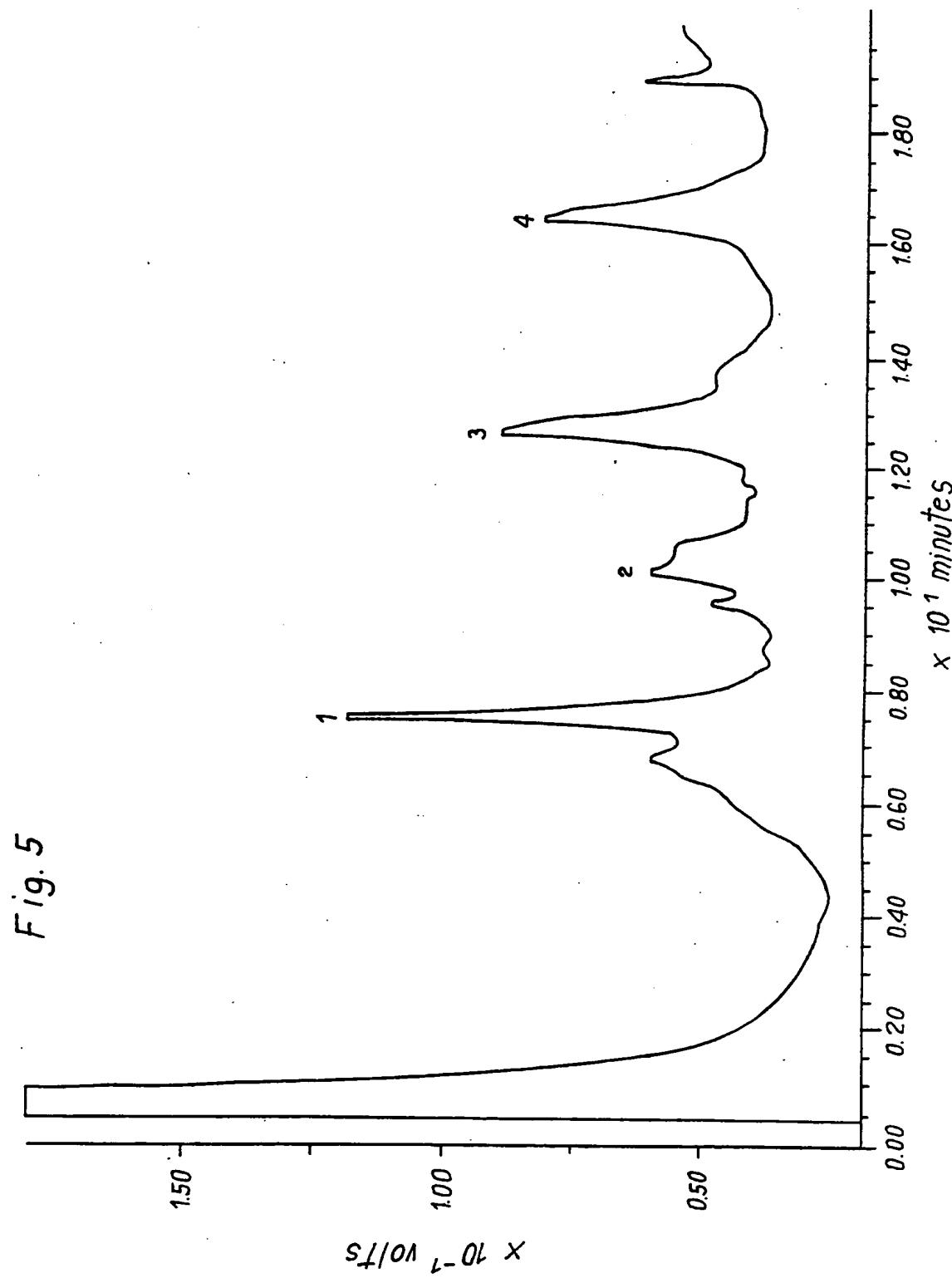


4/12



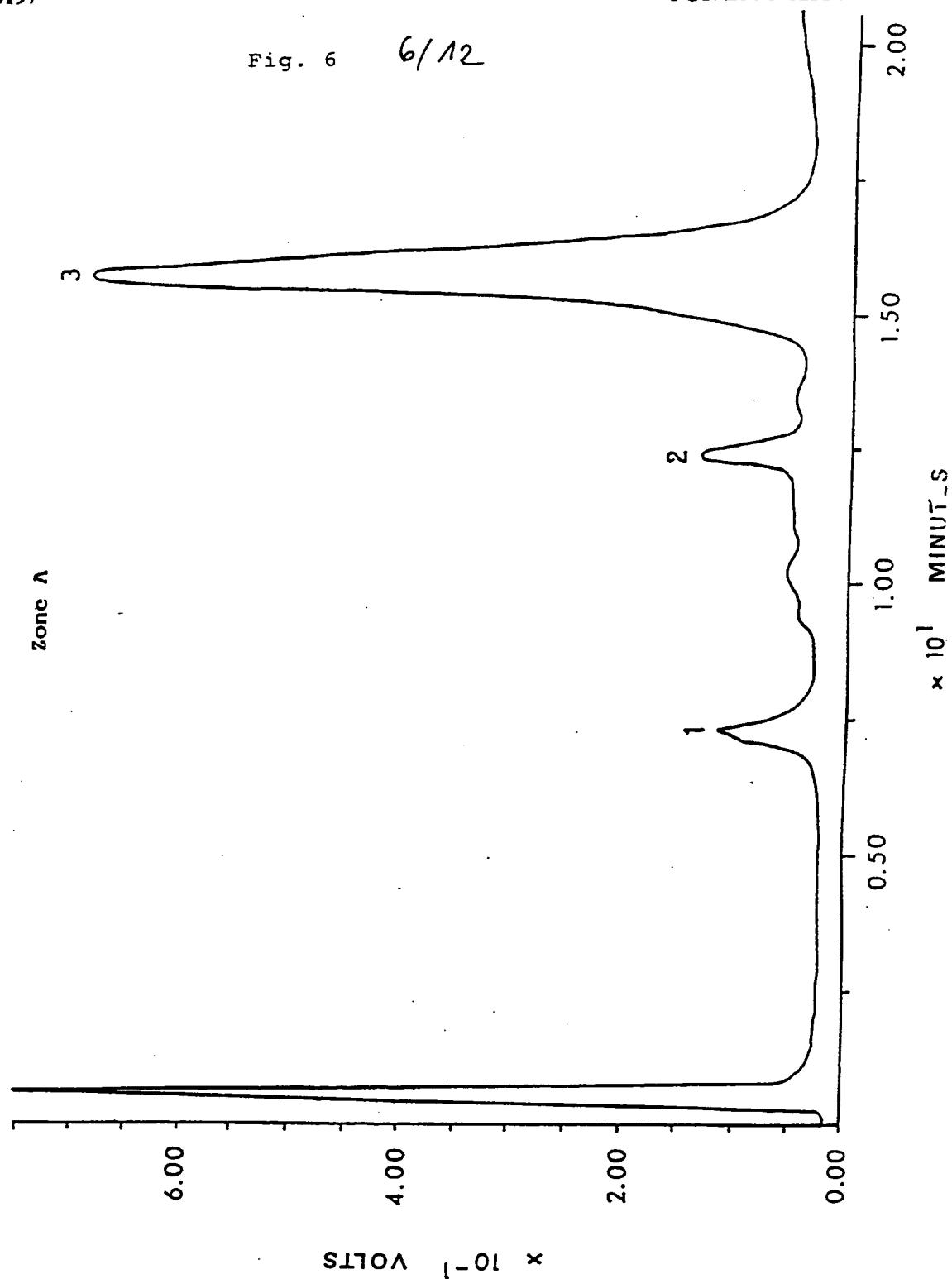
SUBSTITUTE SHEET

5/12



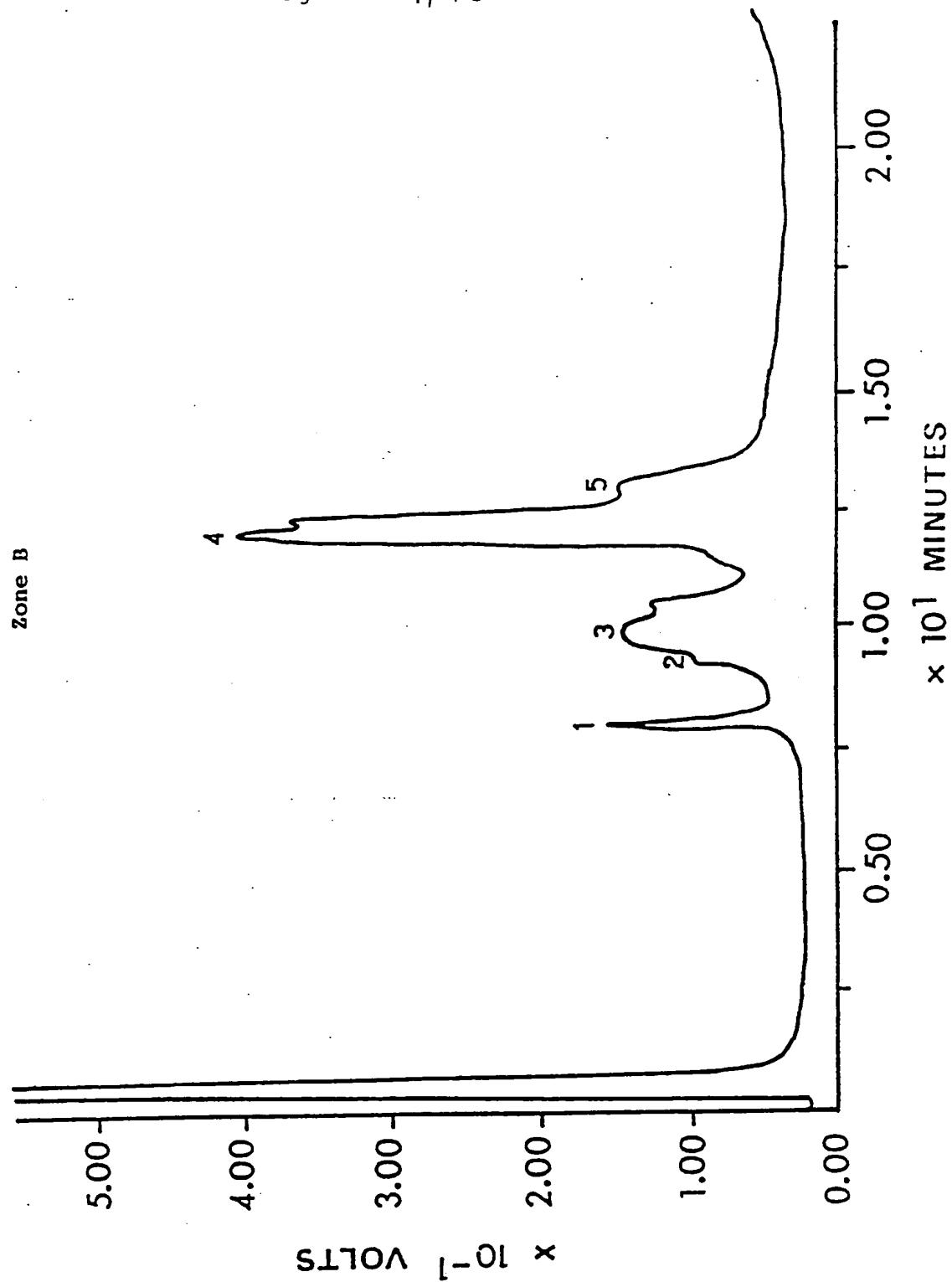
SUBSTITUTE SHEET

Fig. 6 6/12



SUBSTITUTE SHEET

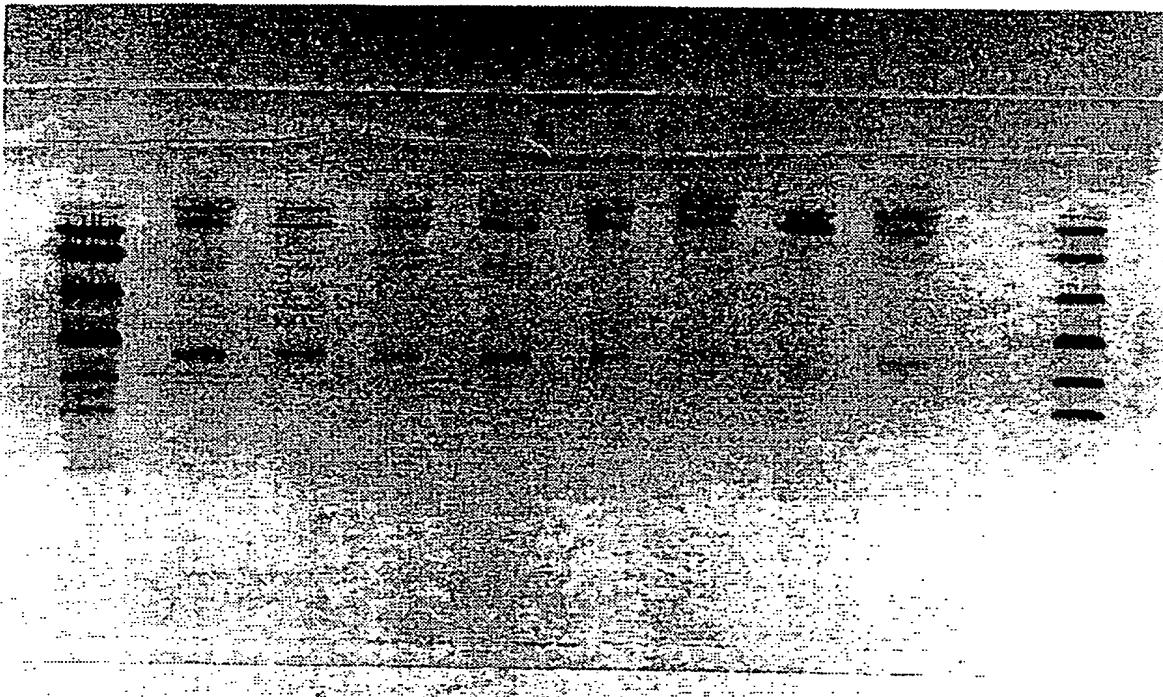
Fig. 7 7/12



SUBSTITUTE SHEET

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ST 1    1    2    3    4    5    6    7    8    ST 2



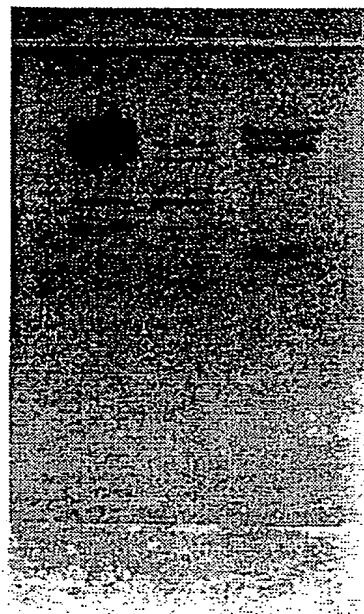
ST 1 = Low molecular weight prestained

- 1 = LGE estr. 1
- 2 = LGE lot. 1/90
- 3 = LGE lot. 2/90
- 4 = LGE lot. 3/91
- 5 = LGE lot. 4/91
- 6 = LGE lot. 5/91
- 7 = NORMAL HUMAN LIVER
- 8 = LGE lot. 6/91

ST 2 = Low molecular weight

9/12

1      2      3



SDS PAGE on PAA 8/18

L.G.E.:

- 1= zone A unbound conA-Sepharose
- 2= zone A bound conA-Sepharose
- 3= lot. 4/91

10/12

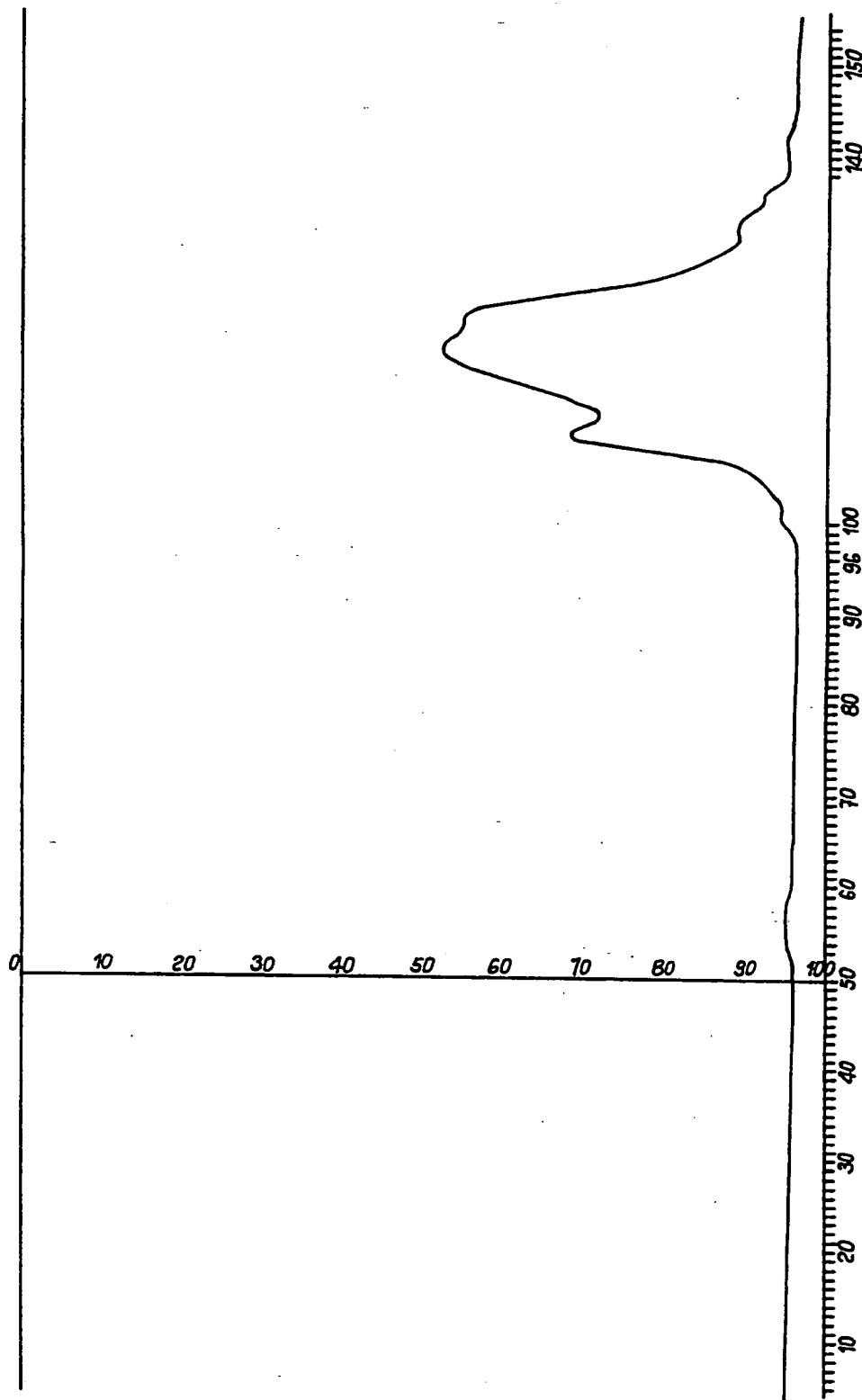
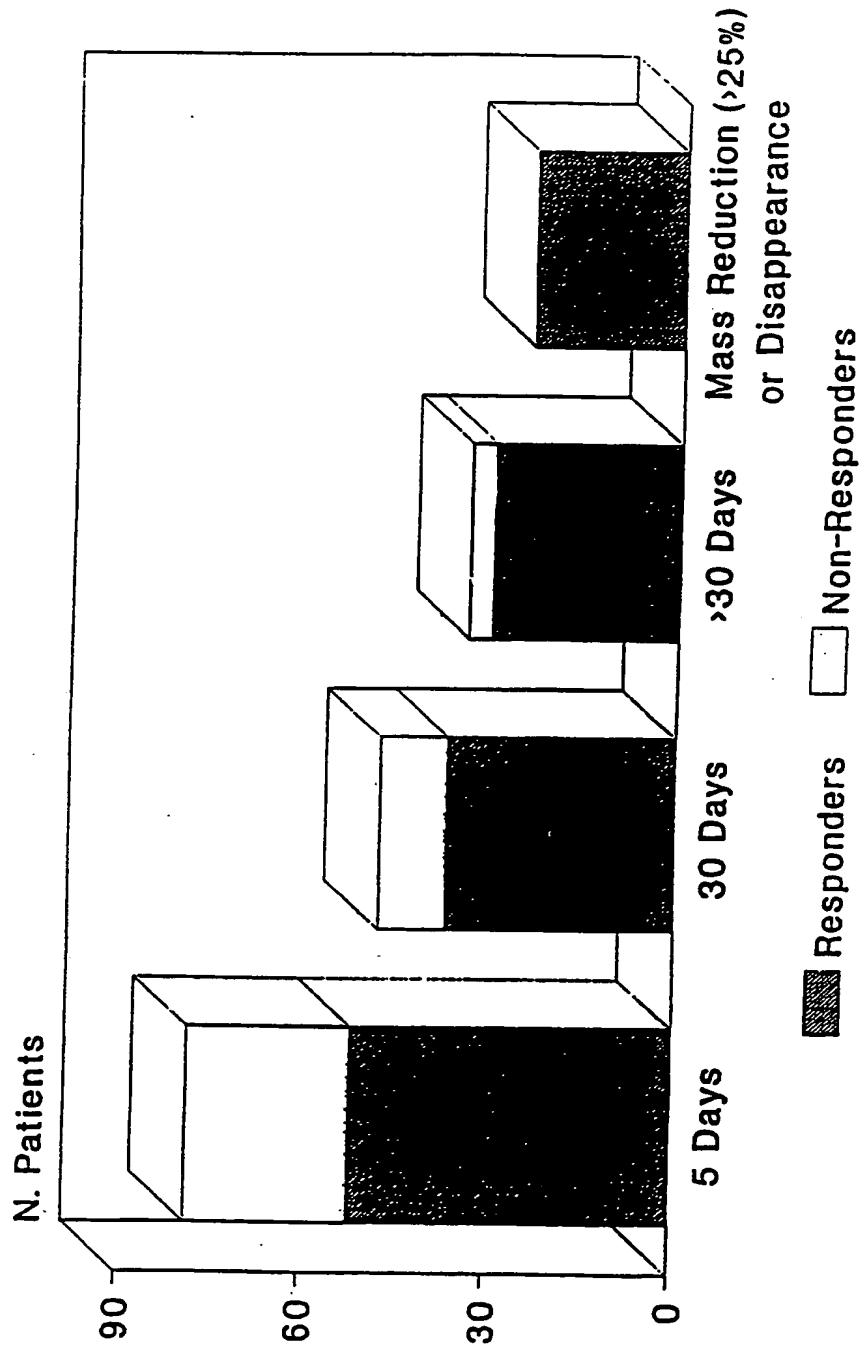


Fig. 10

# L.G.E. THERAPY

## Responders vs Non-Responders

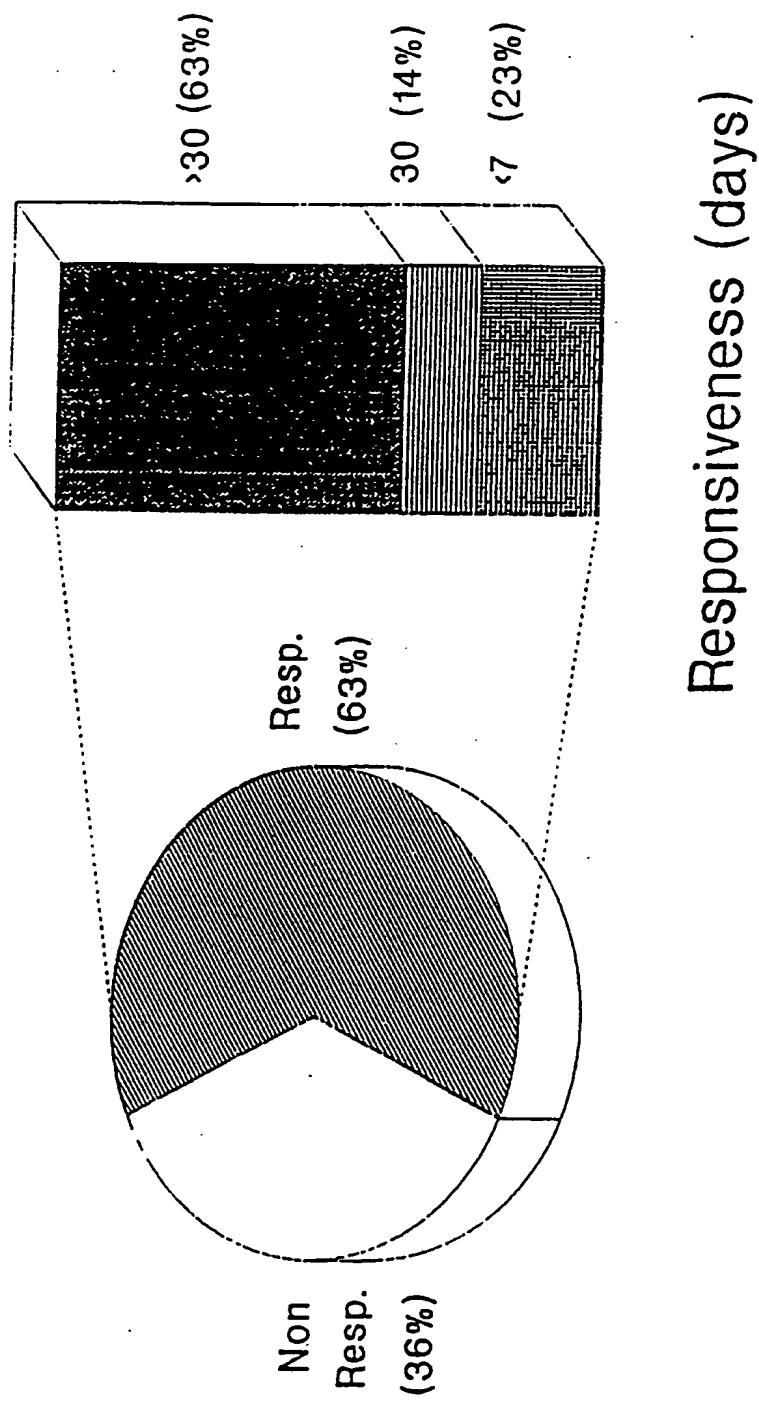


# L.G.E. THERAPY

WO 92/10197

PCT/EP91/02354

12/12  
Fig. 12



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 91/02354

## L. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

**IPC<sup>5</sup> : A 61 K 35/00, A 61 K 35/407, A 61 K 35/38, A 61 K 35/60,  
A 61 K 45/05**

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System	Classification Symbols
IPC <sup>5</sup>	A 61 K, C 07 K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT\*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ***	Relevant to Claim No. 14
A	EP, A2, 0 210 588 (MIZUNO, Den'ichi) 04 February 1987 (04.02.87), see abstract. --	1
A	CHEMICAL ABSTRACTS, vol. 106, no. 23, issued 1987, June 08, (Columbus, Ohio, USA), T. NISHIHARA et al. "Novel antitumor polypeptide pro- duction by human macrophage- like cells." see page 605, the abstract-no. 194 775g, Jpn. Kokai Tokkyo Koho JP 61,280,431 (86,280,431). --	1
A	CHEMICAL ABSTRACTS, vol. 102, no. 3, issued 1985, 21 January, (Columbus, Ohio, USA), T. IKEDA et al. "General methods for isolation of	1

\* Special categories of cited documents: \*\*

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

09 March 1992

Date of Mailing of this International Search Report

26.03.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

*J. TORIBIO*  
NURIA TORIBIO

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	<p>acetic acid- and heat-stable polypeptide growth factors for mammary and pituitary tumor cells." see page 76, the abstract-no. 17 760v, Cell Cult. Methods Mol. Cell Biol. 1984, 2, 217-41.</p> <p>---</p> <p>SCHWEIZERISCHE RUNDSCHAU FÜR MEDIZIN PRAXIS, vol. 79, no. 16, April 17, 1990 A. KAST et al. "Faktor AF2 - die vierte Säule in der Tumortherapie. Dokumentation Nr. 22.", pages 498-502, see totality.</p> <p>----</p>	1,12

## ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

In diesem Anhang sind die Mitglieder der Patentfamilien der im obenge nannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unter richtung und erfolgen ohne Gewähr.

## ANNEX

to the International Search Report to the International Patent Application No.

PCT/EP 91/02354 SAE 53933

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

au rapport de recherche international relatif à la demande de brevet international n°

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
EP A2 210588	04-02-87	EP A3 210588 JP A2 62026226	04-01-89 04-02-87